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(54) Title: THY-1 RECEPTOR AND ITS USE FOR REGENERATING NERVE CELL PROCESSES

(57) Abstract

Purified human Thy-1 receptor, or a purified protein comprising at least a portion of the endogenous human receptor for Thy-1 active in promoting regeneration of a process of a central or peripheral neuron.

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THY-1 RECEPTOR AND ITS USE FOR REGENERATING NERVE CELL PROCESSES

Background of the Invention

This invention relates to methods for enhancing regeneration of mammalian nerve cells in vivo, and to the Thy-1 receptor.

The glycoprotein antigen Thy-1 is expressed on the surface of retinal ganglion cells and on the surface of many neurons not found in the retina. Williams et al., Science 216: 696, 1982, describe the amino acid sequence of Thy-1 glycoproteins. They are analogous to variable regions of immunoglobulin domains. Mason et al., Biochem. J. 187: 1, 1980, describe the production of Thy-1 monoclonal antibody using Thy-1 antigen. Lipton et al. Invest. Opthalmol. Visual. Sci. Supplement 24: 138, 1983, Leifer et al., Soc. Neurosci. Abstract 9: 6, 1983, and Leifer et al. Science 224: 303, 1984, report that retinal ganglions cells of rats, when plated from culture onto glass coverslips previously coated with a monoclonal antibody (2G12 or MRCOX7) against Thy-1, show a substantial increase in the number of solitary perikarya regenerating processes compared with plain glass, glass precoated with collagen, or glass coated with other antibodies.

Summary of the Invention

In a first aspect, the invention features purified human Thy-1 receptor, or a purified protein comprising at least a portion of the endogenous human receptor for Thy-1 active in promoting regeneration of a process of a central or peripheral neuron.

By purified is meant that the Thy-1 receptor is isolated from its natural environment, e.g., on a human cell, and one or more components with which it naturally occurs is reduced in level in comparison to the natural

environment. It is preferred that the receptor be purified to the extent that it forms at least 10% of the protein of a preparation, and even more preferably that it forms the majority of the protein in that preparation. When used for treatment of humans, it is preferred that the Thy-1 receptor be in the form of a homogeneous solution or lyophilized powder of that receptor. That is, the Thy-1 receptor is purified away from all compounds with which it naturally occurs. This term is meant to include purified naturally occurring Thy-1 receptor, but most preferably it refers to synthetic polypeptides forming the biologically active portion of the receptor (i.e., that portion able to promote regeneration of a neuron process), and molecules formed by recombinant genetic engineering techniques. Thus, the term includes polypeptides expressed within a bacterial, mammalian, or other cell, from a strand of DNA which does not naturally occur within that cell, or from DNA under the control of a regulatory sequence with which the Thy-1 receptor-encoding DNA does not naturally occur.

The term human Thy-1 receptor is meant to include not only that protein found naturally occurring in one or more human cells, for example, glial cells, but also any protein found in other organisms which has substantially the amino acid sequence of the active portion of the human Thy-1 receptor, such that administration of the receptor protein of that organism will be effective in promoting regeneration of the process of a central neuron of a human.

The receptor may be purified by a number of techniques. These include isolation of the naturally occurring receptor by immunoaffinity procedures using antibodies against the Thy-1 receptor, or anti-idiotypes (antibodies against Thy-1 antibodies). They also include isolation and purification of cells which naturally

express, or are modified to express, high levels of Thy-1 receptor. Such cells may be formed by genetic engineering techniques to introduce a gene encoding the Thy-1 receptor, and manipulating the engineered cell to cause expression of that gene within the cell. It is also possible to insert secretion sequences, well known to those skilled in the art, upstream of the structural gene encoding Thy-1 receptor, to cause the Thy-1 receptor to be secreted into the medium surrounding the cells and isolated from that medium.

The amino acid sequence of that part of the Thy-1 receptor which is active in promoting regeneration of a process (e.g., an axon or a dendrite) of a central neuron may be determined by standard techniques, e.g., by expressing short fragments of the DNA encoding the receptor, and determining which of the resulting polypeptides are active. This sequence of amino acids can be synthesized by standard chemical procedures well known to those skilled in the art. Such recombinant and synthetic peptides are within this invention.

Also included within the definition of purified Thy-1 receptor is a Thy-1 receptor which is caused to be produced <u>in vivo</u> within a human at a level at which it does not normally occur in that human cell. For example, agents which cause elevated production of the Thy-1 receptor may be administered to the human cells in order to elevate the level of Thy-1 receptor expressed from the naturally occurring gene. Such agents can be identified by standard procedure using the cloned Thy-1 receptor encoding gene and investigating the effect of agents on expression of the gene <u>in vivo</u> or <u>in vitro.</u> In addition, the naturally occurring gene may be modified by transfection of the cell with a virus in order to introduce a gene encoding a Thy-1 receptor, or an agent which modulates expression of the naturally occurring

gene, to increase the level of expression of Thy-1 receptor within that cell.

In a second aspect, the invention features purified nucleic acid encoding human Thy-1 receptor. This nucleic acid is separated from the environment in which it naturally occurs and is generally located on a vector, for example, a plasmid, cosmid, or phage, such that nucleic acid may be manipulated as desired. This nucleic acid includes not only nucleic acid which naturally occurs within a human cell, but modifications (made by standard procedures) of the nucleic acid which cause production of a Thy-1 receptor protein identical in amino acid sequence to a naturally occurring Thy-1 receptor, or having one or more amino acids substituted or deleted without significantly affecting the biological activity of the resulting Thy-1 receptor with regard to its promotion of the regeneration of a process of a central or peripheral neuron. Thus, the nucleotide bases within the nucleic acid may be substituted conservatively or non-conservatively using techniques well known to those skilled in the art. Similarly, nucleic acid closely related to naturally occurring human nucleic acid is encompassed by this invention. Such nucleic acid may be identified by using standard probe screening techniques, where the probe is a portion of at least 15-20 contiguous bases of the nucleic acid encoding human Thy-1 receptor. This probe nucleic acid is used under standard stringent hybridization conditions to identify nucleic acid homologous to that encoding the human Thy-1 receptor. Not all such homologous sequences will encode a Thy-1 receptor protein, but those which do can be identified by standard procedures.

In a third aspect the invention features a method for promoting regeneration of a process of a neuron of a human. The method features providing the purified or

recombinant human Thy-1 receptor described above, and applying that receptor to the neuron in an amount sufficient to promote regeneration of a process of a neuron of a human.

Some potential steps of providing and applying Thy-1 receptor are briefly described above. These steps include provision of a homogenous preparation of Thy-1 receptor protein directly to the neuron to be treated, or application of any other equivalent protein as defined above. Neurons which may be treated in this invention include those of the central nervous system, as well as the peripheral nervous system. Such treatment may include administering the substance in a nerve guide tube, well known to those skilled in the art, via an osmotic minipump, or by use of a slow release pellet, for example, an Elvax pellet. The receptor may also be administered intrathecally, sterotactically or intravitreally (for CNS), or systemically (for PNS).

Alternatively, the level of Thy-1 receptor protein within a cell may be increased by regulation of the naturally occurring Thy-1 receptor gene within that cell, or of a gene which is introduced into the cell by a standard technique. For example, a viral promoter system (e.g., HSV-1 promoter) can be used to cause expression of the Thy-1 receptor in adult glial or other cells, e.g., nerve cells, which no longer, or do not naturally, express the receptor by insertion of that promoter upstream of the natural Thy-1 receptor encoding gene. In addition, a cell line of autologous cells (e.g., glia or other cell types) encoding the Thy-1 receptor can be transfected with such a viral promoter to cause expression of the receptor protein, and these cells transplanted into the CNS or PNS. Fragments of cells or entire cells, e.g., young or passaged astrocytes that

express the Thy-1 receptor, can be introduced sterotactically into the nervous system.

Applicants have found that the Thy-1 receptor of mammals enhances the regeneration of nerve cell processes in vivo in humans. Since Thy-1 is the predominant surface glycoprotein of most nerve cells administration of Thy-1 receptor to cells in vivo should promote significant regeneration of those nerve cells. These receptor proteins are useful in treatment of diseases caused by nerve cell degeneration, for example, dementia or other injury, and also for treatment for peripheral nerve degeneration or severance. For example, after operations known to produce male impotence, administration of the Thy-1 receptor protein at the cut site of the neuron will enhance regeneration of the neuron.

Other features of the invention will be apparent from the following description of the preferred embodiments and from the claims.

Description of the Preferred Embodiments

Thy-1 Receptor

The Thy-1 receptor has been defined above. There follows an example of isolation of one such receptor from human cells, and the isolation of a portion of the gene encoding that receptor. Those skilled in the art will recognize that this example is not limiting to the invention and other methods for isolation of purified Thy-1 receptor will be readily apparent upon reading of this disclosure. Similarly, the complete Thy-1 receptor encoding gene can be readily isolated by standard techniques using the partial clone provided in this application. To this end a deposit has been made of a clone including 500 bases encoding the Thy-1 receptor. This deposit has been made in the American type culture collection and applicants assignee, Children's Medical

Center Corporation of Boston, acknowledges its responsibility to replace this culture should it die before the end of the term of the patent issued hereon, five years after the last request for a culture, or 30 years, which ever is longer, and its responsiblity to notify the depositry of the issuance of such patent, at which time the deposit will be made irrevocably available to the public. Until that time the deposit will be made available to the Commission of Patents under the terms of 37 C.F.R. sections 1-14 and 35 U.S.C. section 112. The deposit is assigned the number _____ and was deposited on August 9, 1989.

In the example to be described, anti-idiotypic antibodies to Thy-1 antibodies were used to screen a cDNA library of human DNA. The anti-idiotypic antibodies against Thy-1 antibodies were characterized by Pillemer et al. J. Experimental Medicine, 153:106, 1981, hereby incorporated by reference herein. These were obtained and purified according to the methods described by Pillemer et al. These anti-idiotypic antibodies are not essential to the present invention since other anti-idiotypic antibodies against Thy-1 can be used to identify clones, as described below. Applicant believes that the anti-idiotypic antibodies used in the present invention are available from Pillemer et al, or commercially available from Sigma (St. Louis) or Hazelton Labs. (PA).

The anti-idiotypic antibodies TEPC-15, HOPC-8, MOPC-603, and MOPC-167 of Pillemer et al. were purified from ascitic fluid over an anti-IgA affinity column. Media surrounding the cells was centrifuge-filtered and passed through an Amicon concentrator. This was stored at -80° until use. The ascitic fluid was then passed over an anti-IgA affinity column, and the eluate dialyzed and concentrated against 0.5 M NaCl, 0.1 M $NaHCO_3$.

Example 1: cDNA encoding part of human Thy-1 receptor.

A fetal human brain cDNA library was constructed in lambda gt11. To accomplish this, cDNA was synthesized from 10 mg of polyadenylated RNA by a modification of the method of Gubler et al., Gene 25:263, 1983. A double-stranded cDNA was treated with EcoR1 methylase, ligated to EcoR1 linkers, cleaved with EcoR1, and passed over a Sepharose CL4-B column to remove excess linkers. The cDNA was ligated to lambda gt11 DNA which had been pre-ligated, digested with 100-fold excess of EcoR1, and phosphatased using one unit of calf intestinal alkaline phosphatase (Boehringer Mannheim) per 70 mg of phage DNA. Approximately 500 ng of cDNA was ligated to 50 ng of lambda gt11 arms. The DNA was packaged according to standard protocols and amplified in E. coli Y 1088 on tryptone plates (1% Bacto-tryptone, 0.5% NaCl, 1.2% agar, 2 mM $MgCl_2$) in tryptone top agar (0.8% agar). To screen the library, the phage was grown on Y1090 at a density of 100,000 per 150 mm plate. Standard protocol was followed through incubation of the filters in the primary (anti-idiotype) antibodies in Tris buffer with 1% goat serum. Filters were then washed twice is Tris buffer containing 0.1% Triton X-100, incubated in peroxidase-conjugated goat anti-mouse IgA second antibodies, washed in 50 mM Tris, pH 7.4, and visualized with diaminobenzidine and hydrogen peroxide. A positive plaque was then cloned and used in a Northern blot analysis for hybridization to mouse RNA obtained from cultures of astrocytes.

A preliminary screening of the library using the anti-idiotype antibodies TEPC-15 and HOPC-8 revealed a single positive clone consisting of approximately 500 bases of cDNA, designated TR1.

Rescreening of the brain library with the 500 base clone identified two two kilobase and three three

kilobase clones to which the 500 base clone hybridizes. These clones are of sufficient length that they may represent the entire coding region of the receptor. The mRNA detected on Northern blots with TR1 is 6 kb in size. However, much of it may consist of nontranslated sequences. In Western blots, 30-60 kd bands were detected, suggesting that the Thy-1 receptor requires only about two kilobases to encode the protein.

The above clone of Thy-1 receptor can be used to identify other candidate receptor clones to insure that a full length Thy-1 receptor clone is obtained. This clone may be manipulated by standard procedures to determine the DNA sequence of the clone, and thence the amino acid sequence of the Thy-1 receptor. Once these sequences are determined the Thy-1 receptor gene can be manipulated into any standard expression vector to cause expression of the Thy-1 receptor protein from any other number of standard expression cells. The expressed Thy-1 receptor protein is then isolated and purified by standard techniques. A Thy-1 receptor-encoding gene can also be transfected into mammalian cells, for example, by using recombinant clones in the DO vectors, such as DOL and/or DOJ, which contain the polyoma early region, or pBR322. These retro virus constructs are used to overexpress the protein in a mammalian cell line that does not normally express Thy-1 receptor. For example, NIH 3T3 cells can be used. In this method a sodium phosphate buffer at pH 6.95 is mixed with a calcium DNA solution, and the calcium phosphate DNA precipitate formed allowed to precipitate gradually onto the cells to provide a high efficacy of stable transformation. Those cells transformed are expanded by standard procedure. An example of an eukaryotic expression library includes the pH3M vector, having an SV40 origin of replication, an M13 origin, a SupF marker, and a convenient cloning site.

Cells expressing the Thy-1 receptor are selected by panning (i.e., by their adherence to dishes coated with anti-idiotypic antibody or with purified Thy-1). DNA is isolated from adherent cells and used to transform MC1061/p3 cells, amplified, and used to transfect COS cells for a second round of enrichment. Standard methods can be used to produce variants of the DNA clones, which may include variants of the Thy-1 receptor at the amino acid level.

Example 2: Affinity purification of Thy-1 receptor

Thy-1 receptor may be purified by affinity column purification. The Thy-1 receptor is purified by passage over s Thy-1-Sepharose 4B column. Thy-1 is coupled to the cyanogen bromide activated Sepharose and either astrocyte cultures or whole brain preparations solubilized in detergent (Nonidet P-40 or sodium deoxycholate) passed over the Sepharose-Thy-1 column, and then equilibrated with buffer (1M Tris HCl, pH 7.4). Elution of Thy-1 receptor is with acidic and basic buffers (e.g., 0.1 M glycine HCl, pH 2). The resulting fractions are assayed for protein, and those fractions containing protein assayed for Thy-1 receptor. The receptor is identified by standard SDS polyacrylamide gel electrophoresis, or by immunoblot analysis. Alternatively, anti-idiotypic antibodies such as those discussed above, or monoclonal antibodies to Thy-1 receptor, may be used in its detection.

Methods

In order to use the above described Thy-1 receptor, any number of methods may be utilized as discussed above.

Example 3: Promotion of regeneration with Thy-1 receptor.

One method of this invention includes providing a supply of Thy-1 receptor protein immobilized on a solid support. This support is placed in proximity to a mammalian neural process in a living mammal to promote growth and regeneration of the process. The method of the invention is particularly applicable to neural processes including ganglion cell bodies, such as retinal ganglion cell bodies, but can also be used to enhance regeneration of nerve cell processes in the peripheral as well as the central nervous system.

The term "solid support" includes both rigid materials such as glass, synthetic plastics, and the like on the surface of which a layer or coating of the monclonal antibody is bonded, and also soft gels penetrable by the regenerating nerve process such as collagen, fibrin, fibrinogen, blood clot, or laminin throughout which the monoclonal antibody is dispersed and bonded so that it is immobilized. A supply of Thy-1 receptor immobilized in a soft gelatinous mass such as a proteinaceous gel, for example collagen gel, enclosed within an open ended tube or hollow cylinder of glass or synthetic plastic which serves as a guide for the regenerating processes, the latter advancing through the center of the tube, can also be used. The synthetic plastic used for the tube or hollow cylinder can be any nontoxic material to which antibodies bond, such as polyethylene, polypropylene, etc., but is preferably a bioresorbable material such as that described in Nyilas, U.S. Patent 4,481,353. The guide filled with proteinaceous gel on which Thy-1 receptor is immobilized is implanted so as to bridge the gap between the ends of a damaged or severed process to enhance regeneration and extension of the process through the center of the tube spaced from the synthetic plastic wall. The following example is intended to illustrate more fully the nature

of the invention without acting as a limitation upon its scope. It is understood that this example demonstrates the utility of Thy-1 receptor in rats but that naturally or traumatically induced defects in neural processes can also be treated in this manner.

Male Sprague-Dawley rats can be utilized in these studies. Four animals serve as an unoperated control and three animals receive an intracranial optic nerve transection alone. In eight animals, one optic nerve is transected intracranially and then a nerve guide tube implanted to bridge the transected nerve. To achieve this, a small craniotomy is made in the frontal lobe and carefully aspirated to expose a 2 to 3-mm segment of one optic nerve intracranially. The pial plexus of capillaries together with the anterior cerebral artery are gently retracted medially and the nerve transected. Such an intracranial nerve transection spares the blood supply to the retina.

After hemostasis is achieved, a 1.5-mm length (0.95 mm o.d, 0.75 mm i.d.) of the nerve guide is inserted. The nerve guides used in these experiments are fabricated as polymers of synthetic poly D,L-lactates with 2% triethyl citate as a plasticizer, as described in U.S. Patent 4,481,353.

The experimental animals are divided into three groups. In one group (two rats) empty nerve guide tubes are implanted. In the second group (three rats) the nerve guides lumen is filled with a collagen matrix (Vitrogen, Flow Laboratories, diluted in 0.1 M phosphate buffer to a final concentration of 2.48 mg/ml) containing a 0.5 mg/ml fibrinogen (bovine, Cal Biochem) immediately before implantation. The third group (three rats) receive nerve guides containing Thy-1 receptor, in addition to the collagen and fibrinogen.

In all animals, both the proximal and distal nerve stumps are inserted into the guide. At the time the nerve guides are implanted, all experimental animals receive bilateral superior cervical ganglionectomies to remove the sympathetic innervation to the head. This procedure is necessary to rule out the ingrowth of peripheral sympathetic fibers, a process which has been shown to occur after indirect and direct lesions to the CNS.

Four weeks postoperatively the animals are deeply anesthetized and perfused through the heart with 200 ml heparinized saline, followed by 500 ml 1% paraformaldehyde plus 3% glutaraldehyde, followed by 200 ml 0.1 M phosphate buffer. All perfusion solutions are made up in 0.1 M phosphate buffer. The nerve guide is dissected out as one piece, immersed in 1% osmium tetroxide, and processed for embedding in plastic (DER, Ted Pella, Inc.). One micrometer transverse sections are cut from the proximal, middle, and distal portions of the nerve guide and stained with toluidine blue. "Proximal" refers to sections across the part of the nerve guide closest to the eye.

Data are collected with a computer-controlled light microscope at a final magnification of 1600X. Blood vessels are identified and their luminal profiles entered on-line from a digitizing tablet to a display terminal and then to a VAX 11/780 computer (Digital Equipment Corp.) for further numerical and statistical analysis. For each cross-sectioned sample the tissue cable areas, the number of blood vessels, and the area of each blood vessel is obtained. This data allows determination of whether the Thy-1 receptor is active in promoting neural process growth.

This procedure can also be used to determine the use of various forms of Thy-1 receptor, or fragments

thereof. For example, recombinant Thy-1 receptor can be expressed by standard procedure and tested for its efficacy in this method. By expression of parts of the Thy-1 receptor-encoding DNA that portion having the desired activity in this assay can readily be determined.

One other method for measuring the use of a Thy-1 receptor is the retinal culture procedure of Leifer et al, 1984, supra. Generally, rat and mouse retinal ganglion cells are identified with fluorescent dyes that are retrogradely transported to the ganglion cell bodies after injection into the superior colliculus and lateral geniculate body, to which the ganglion cells project. Fluorescently-tagged Thy-1 antibodies are specific among retinal cells in vitro for the ganglion cells. Double labelling experiments show virtually complete overlap between the two marking methods, ensuring the validity of either for identifying the ganglion cells. Ganglion cells are dissociated from the retinas of 4 day-old to adult rats or mice under anesthesia using papain (10 units/ml, Cooper Biomedical, Inc.) and mild mechanical trituration. The dissociated retinal cells are plated in culture using minimal essential media and 5% rat serum (for rat cultures) or fetal calf serum (for mouse cultures). For rat cultures, Long-Evans rats are used, and, for mouse cultures, C57BL/6 mice.

To measure the regeneration of processes by the ganglion cells, retinal cells from a single dissociation are cultured in multiple dishes (each receiving a 50 µl drop of the same dilution of cells). On the second, third, and fourth days in culture, solitary ganglion cells are measured for the diameter of the soma, and each process is measured using a computer graphics system.

Other Embodiments

Other embodiments are within the following claims.

Claims

1. Purified human Thy-1 receptor.

2. Recombinant human Thy-1 receptor.

3. A purified protein comprising at least a portion of the endogenous human receptor for Thy-1, which portion is active in promoting regeneration of a process of a central or peripheral neuron of a human.

4. Purified nucleic acid encoding human Thy-1 receptor.

5. A method for promoting regeneration of a process of a neuron of a human, comprising the steps of:

providing purified or recombinant human Thy-1 receptor, and

applying said receptor to said neuron in an amount sufficient to promote regeneration of a process of a neuron of a human.

INTERNATIONAL SEARCH REPORT

International Application No PCT/US90/04478

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [3]

According to International Patent Classification (IPC) or to both National Classification and IPC

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U.S. CL.: 530/350, 399

II. FIELDS SEARCHED

Minimum Documentation Searched [4]

Classification System	Classification Symbols
U.S.	530/350, 399

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [5]

III. DOCUMENTS CONSIDERED TO BE RELEVANT [14]

Category *	Citation of Document, [16] with indication, where appropriate, of the relevant passages [17]	Relevant to Claim No. [18]
A	SCIENCE, Volume 224, issued 20 April 1984, Leifer, "Monoclonal antibody Thy-1 Enhances Regeneration of Processes by Rat Retinal Ganglion Cells in Culture p. 303-306.	1-3, 5
A	SCIENCE, Volume 216, issued 14 May 1982, Williams et al., "Neuronal Cell Thy-1 glycoprotein: Homology w/Immunoglobulin", p. 696-703.	1-3,5

* Special categories of cited documents: [16]

"A" document defining the general state of the art which is not considered to be of particular relevance

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IV. CERTIFICATION

Date of the Actual Completion of the International Search [2]	Date of Mailing of this International Search Report [2]
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ISA/US	SHELLY F. GUEST